Table 2. Area Under the ROC Curves, Sensitivity, Specificity, and Negative and Positive Predictive Values of Non-Virologic Responses

Variables	Az	95% CI	Cut-off	Sensitivity	Specificity	NPV[a]	PPV[b]
RIG-I	0.89	0.78–0.95	0.68	0.80	0.87	0.92	0.70
MDA5	0.92	0.86–0.98	0.84	0.82	0.89	0.93	0.74
LGP2	0.76	0.63–0.90	1.03	0.65	0.72	0.85	0.46
RIG-I/Cardif	0.91	0.84–0.99	0.88	0.75	0.91	0.91	0.75
RIG-I/RNF125	0.81	0.69–0.93	1.05	0.82	0.62	0.91	0.43
ISG15	0.91	0.85–0.97	0.36	0.90	0.81	0.96	0.64
USP18	0.90	0.84–0.96	0.67	0.90	0.83	0.96	0.67

[a]NPV, negative predictive value.
[b]PPV, positive predictive value.

we determined the basal protein expression levels of Cardif in the liver in NVR and SVR patients. Western blot analysis demonstrated a single Cardif product in all samples (Figure 4*A*). Similar to Cardif mRNA expression, mean Cardif expression in NVR patients was significantly lower than that in SVR (Figure 4*B*, $P = .01$). The cleavage product of Cardif, which has been reported by Loo et al,[23] was not detected in our analyses.

Transcriptional Responses to PEG-IFN-α-2b and Ribavirin Therapy in PBMC

Sequential analysis in response to PEG-IFN-α-2b and ribavirin demonstrated a rapid and strong induction of RIG-I, ISG15, and USP18 mRNA expression, which peaked 8 hours after PEG-IFN-α-2b administration (Figure 5). A greater fold change of these peak inductions was observed in SVR patients compared with NVR patients, although statistical significance was not achieved. In marked contrast, RNF125 expression profile in response to PEG-IFN-α-2b was triphasic, and consisted of (1) rapid and strong suppression peaked at 8 hours after administration, (2) increased 1.5- to 2-fold above baseline level during 24–48 hours after the administration, and (3) gradually decreased to baseline level (Figure 5). The rapid suppression and subsequent increase following PEG-IFN-α-2b administration tended to have a greater fold change in NVR patients compared with those in SVR patients. In contrast from RIG-I, ISG15, USP18, and RNF125, Cardif expression profile was relatively constitutive, and transcriptional response to PEG-IFN was weak (Figure 5).

Discussion

In the present study, we found that baseline expression levels of intrahepatic viral sensors and related regulatory molecules were significantly associated with the final virologic outcome in patients with chronic hepatitis C who were treated with PEG-IFN-α-2b and ribavirin combination therapy: up-regulation of RIG-I, MDA5, LGP2, ISG15, and USP18 and lower expression of Cardif and RNF125 could predict nonresponse to subsequent treatment with PEG-IFN-α-2b and ribavirin. The positive predictive value of a high ratio of expression of RIG-I to Cardif (>0.88) for NVR was the highest at a value of 0.75, and the negative predictive values of high expression of ISG15 (>0.36/internal control) and USP18 (>0.67/internal control) were the highest at values of both 0.96. These data may be of use in predicting clinical responses to the PEG-IFN-α and ribavirin combination before initiating therapy.

Table 3. Multivariate Analysis for the Factors Associated With Non-Virologic Response

Variable	Odds ratio	95% CI	*P* value
RIG-I/Cardif Ratio (by 0.1)	1.5	1.1–2.1	.008
RIG-I/RNF125 Ratio (by 0.1)	1.2	1.0–2.5	.1
ISG15 (by 0.1/internal control)	1.5	1.1–2.0	.01
Age (by 1 y)	1.0	0.9–1.1	.6
Platelet count (by $1 \times 10^4/\mu L$)	1.2	0.9–1.5	.07

Previously, large randomized controlled trials identified several pretreatment factors associated with the final virologic outcome, such as genotype, HCV RNA level, degree of fibrosis, age, body weight, ethnicity, and steatosis.[24] However, these findings lead us to believe that predicting the final virologic response before initiating PEG-IFN-α and ribavirin is difficult. Indeed, only age and platelet count were associated with the outcome in our patients with genotype 1b and a high viral load. Currently, the final response can be gauged only after treatment has been initiated. Although an early viral response at 12 weeks suggests the eventual outcome with 60%–90% accuracy,[25] a 12-week regimen is associated with adverse effects and is expensive. Therefore, this study investigated the baseline expression of genes involving innate immunity that may have significant effects on clinical outcomes.

In the present study, we demonstrated that RIG-I and MDA5 were inducible upon HCV infection and that expression of these intrahepatic positive viral sensors was up-regulated in NVR. In vitro studies have suggested that RIG-I and MDA5 play a pivotal role in the regulation of IFN production and augment the production of IFN via an amplification circuit. These results suggest that expression of RIG-I and MDA5 and related amplification system may be up-regulated by endogenous IFN at a higher baseline level in NVR patients. However, HCV elimination by subsequent exogenous IFN is insufficient

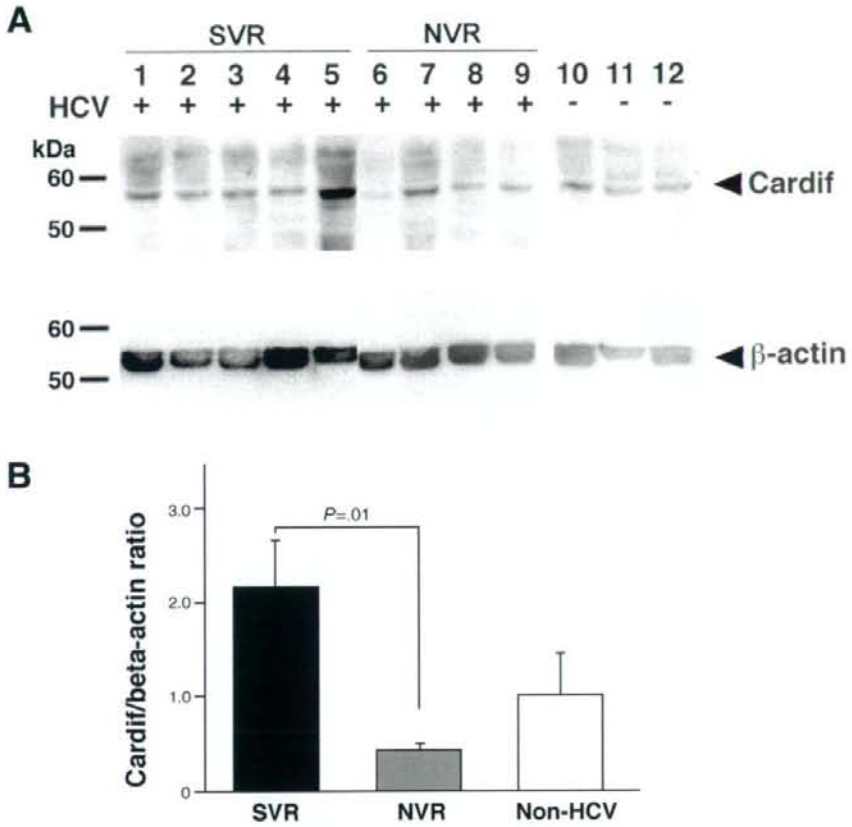

Figure 4. (*A*) Western blot analysis. Five lanes were SVR (*lanes 1–5*), 4 lanes were NVR (*lanes 6–9*), and 3 lanes were non-HCV control (*lanes 10–12*). Specific bands for Cardif and β-actin are indicated by *arrows*. (*B*) Expression level of Cardif protein normalized to β-actin in the liver biopsy specimens according to ultimate treatment response. *Error bars* indicate the standard error.

in these patients, suggesting that NVR patients may have adopted a different equilibrium in their immune response to the virus. In contrast to the expression of RIG-I and MDA5, Cardif mRNA, which was expressed in a relatively constitutive fashion, was significantly lower in NVR. Our ROC analysis highlights that lower expression of Cardif relative to that of RIG-I was one of the strongest predictors for NVR. Moreover, Western blot analysis further confirmed the down-regulation of Cardif in NVR patients, as demonstrated by its protein level. Because Cardif is one of the substantial target molecules of HCV evasion,[11,20] it is likely that Cardif expression is suppressed by HCV with resistant phenotype or is inadequate in NVR patients. Loo et al have demonstrated a Cardif cleavage product in 2 of 4 liver tissue samples of chronic HCV infection.[23] In our study, however, the Cardif cleavage product was not detected, presumably because the product could be unstable in vivo, resulting in rapid degradation. Although further studies are necessary to elucidate mechanisms of Cardif down-regulation, our findings of lower expression of Cardif in NVR suggested that the status of Cardif expression in the liver might have a significant effect on the ultimate outcome of antiviral treatment.

The antiviral effect brought by RIG-I/Cardif signaling is regulated by the coordination of negative and positive regulators. It has been shown that RNF125 functions as a negative regulator of RIG-I/Cardif signaling. RNF125 is an ubiquitin E3-ligase with activity against protein containing CARD domains, such as RIG-I, MDA5, and Cardif, and these ubiquitinated molecules undergo proteasomal degradation. In contrast, RNF125 do not have negative function against LGP2, a negative regulator of RIG-I signaling, because LGP2 lacks CARD domain. In contrast to RIG-I, RNF125 expression was rapidly suppressed by exogenous IFN; therefore, observed lower basal hepatic level of RNF125 in NVR could be explained by the suppressive effect of endogenous IFN, which may be up-regulated in NVR patients. Hence, RNF125 may constitute a negative regulatory circuit for IFN production and is responsible for responsiveness to PEG-IFN and ribavirin therapy.

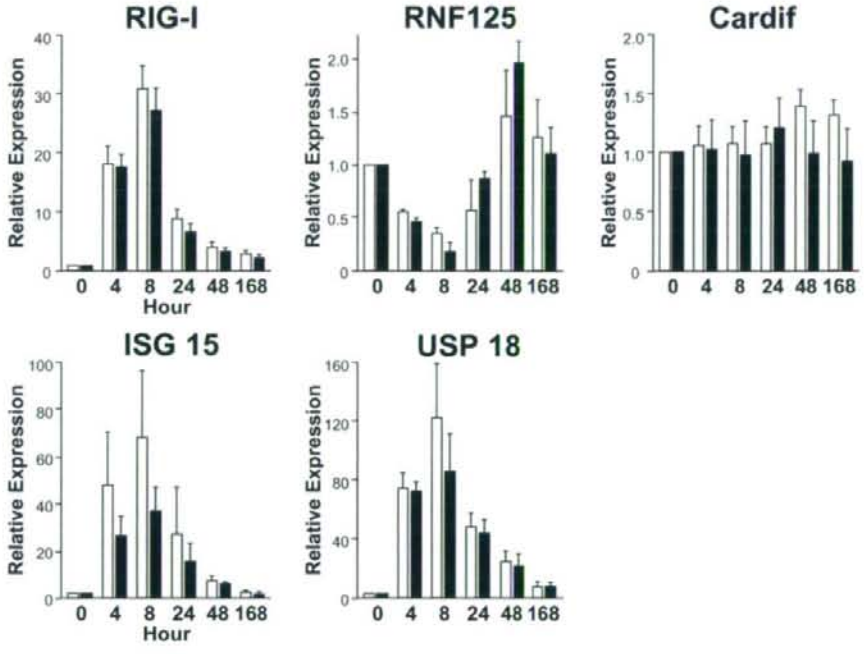

Figure 5. Transcriptional responses during PEG-IFN-α-2b and ribavirin therapy in PBMC (n = 14). *Open columns* indicate SVR (n = 7), and *solid columns* indicate NVR (n = 7). *Error bars* indicate the standard error. The *P* values determined by Mann–Whitney *U* test between 2 groups at 8 hours were as follows: RIG-I, *P* .3; RNF125, *P* .3; Cardif, *P* .7; ISG15, *P* .3; USP18, *P* .2.

It has been shown that RIG-I function is modified by ISG15 via ISGylation.[17] Consistent with our data, Chen et al identified 18 genes, including ISG15 and USP18, whose expression differed between responders and nonresponders.[26] Interestingly, a recent study has shown that USP18 negatively regulates IFN signaling independently of its isopeptidase activity toward ISG15 by binding to the IFNAR2 receptor subunit and blocking the interaction between Janus kinase and the IFN receptor.[27] Moreover, the siRNA knockdown of USP18 in human cells has consistently been shown to potentiate the ability of IFN to inhibit HCV RNA replication.[28] Therefore, USP18 is suggested as a novel in vivo inhibitor of signal transduction pathways that are specifically triggered by type I IFN. Consistent with a role for USP18 in down-regulating the antiviral IFN response, we confirmed that up-regulation of USP18 was one of the factors predicting a lack of response to treatment with IFN.

The mechanism underlying the association of gene expression involving innate immunity with resistance to therapy is not well understood. Our human study with HCV patients treated by PEG-IFN and ribavirin highlights RIG-I/Cardif, RIG-I/RNF125, and ISG15/USP18, which is partly responsible for the clinical responsiveness to antiviral therapy. RIG-I signaling by viral pathogens may affect a wide variety of responses in not only innate but also acquired immunity. Our study is the first to demonstrate the potential relevance between molecules involving innate immunity and the clinical response to antiviral therapy.

In addition, sequential analysis of expression profile during PEG-IFN-α-2b and ribavirin treatment was also performed in this study. Lanford et al demonstrated transcriptional response to IFN-α in chimpanzee by genome microarray analysis, which included RIG-I, ISG15, and USP18.[29] An association of transcriptional response with early phase of virologic response has been also reported in PBMC or liver biopsy specimen.[30-32] We recently reported that the transcriptional double-stranded RNA-activated protein kinase response during treatment with PEG-IFN-α-2b and ribavirin was associated with the ultimate clinical response.[30] Similarly, the present study demonstrated a strong and rapid increase of RIG-I, ISG15, and USP18 mRNA in response to clinical PEG-IFN treatment especially in SVR patients, although few patients were available to achieve statistical significance between SVR and NVR. In marked contrast, transcriptional response of RNF125 exhibited a triphasic pattern. Rapid suppression seen in the first phase was presumably because of a negative regulatory effect of IFN. However, increase of RNF125 mRNA in the second phase, which tended to be grater in NVR, may be responsible for inhibiting RIG-I expression seen 8–48 hours after PEG-IFN-α-2b administration. Although limitations includ-

ing the use of PBMC and small sample size still deserve mention, the sequential expression profile during treatment may provide further valuable information regarding the prediction of the clinical response to the therapy and the mechanism of action of antiviral treatment.

In the present study, we have included patients with genotype 1b because it is imperative to designate a virologically homogeneous patient group to associate individual treatment responses with different gene expression profiles that direct innate immune responses. We have preliminarily studied genotype 2 patients and found that Cardif and RNF125 gene expression levels in NVR patients were significantly lower than those with SVR patients ($P = .03$ and $P = .04$, respectively) and that RIG-I/Cardif and RIG-I/RNF125 ratios were significantly higher in NVR patients ($P = .02$ and $P = .009$, respectively, see Supplementary Figure 2 online at www.gastrojournal.org). These findings suggest that the differences in gene expression profiles between SVR and NVR were almost identical to those demonstrated in patients with genotype 1b. However, the correlation between treatment responses in all the genotypes and the different status of innate immune responses needs to be explored. Further studies may be necessary to clarify this issue.

In conclusion, the results of the present study offer potentially important clinical implications for patients with chronic hepatitis C who are treated with PEG-IFN-α and ribavirin. Quantifying hepatic gene expression of the RIG-I/Cardif system, including its regulators before treatment, is useful in identifying patients who are at a higher risk for NVR. The data from these assays can provide valuable information that may influence the decision about the treatment strategy in each individual patient. Finally, this clinical human study demonstrates the potential relevance of the molecules involving innate immunity to the clinical response to therapy. Our data will help understand the pathogenesis of HCV resistance and development of new antiviral therapy targeted toward the innate immune system.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2008.02.019.

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Targeting Lipid Metabolism in the Treatment of Hepatitis C Virus Infection

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Recently, microdomains of organelle membranes rich in sphingomyelin and cholesterol (called "lipid rafts") have been considered to act as a scaffold for the hepatitis C virus (HCV) replication complex. Using the HCV cell culture system, we investigated the effect of myriocin, a sphingomyelin synthesis inhibitor, on HCV replication. We also investigated the combined effect of myriocin with interferon (IFN) and myriocin with simvastatin. Myriocin suppressed replication of both a genotype 1b subgenomic HCV replicon (Huh7/Rep-Feo) and genotype 2a infectious HCV (JFH-1 HCV) in a dose-dependent manner (for subgenomic HCV-1b, maximum of 79% at 1000 nmol/L; for genomic HCV-2a, maximum of 40% at 1000 nmol/L). Combination treatment with myriocin and IFN or myriocin and simvastatin attenuated HCV RNA replication synergistically in Huh7/Rep-Feo cells. Our data demonstrate that the sphingomyelin synthesis inhibitor strongly suppresses replication of both the subgenomic HCV-1b replicon and the JFH-1 strain of genotype 2a infectious HCV, indicating that lipid metabolism could be a novel target for HCV therapy.

Hepatitis C virus (HCV) is a major etiologic agent of liver diseases, affecting 170 million people worldwide [1]. Fifty-five percent to 85% of acute infections become persistent [2], and at least 20% of patients with chronic HCV infection progress to cirrhosis within 20 years [3]. With therapeutic advances, including the recent combination of pegylated interferon (IFN) plus ribavirin, half of patients can achieve a sustained virologic response [4]. However, the remaining half cannot clear the virus, demonstrating a strong need for HCV-specific therapies.

Positive-strand RNA viruses replicate intracellularly on certain membrane structures, including the endoplasmic reticulum [5], the Golgi apparatus [6], endosomes, and lysosomes [7]. During replication, RNA viruses form distinct replication complexes made of several membrane compartments and viral proteins [8]. In HCV, the membranous web (consisting of vesicles in a membranous matrix) has been described in the cellular matrix of HCV replicon–harboring cells [9, 10]. This membranous web is considered to be the HCV replication complex, consisting of viral and host proteins.

Recent studies suggest that the HCV replication complexes are formed on lipid rafts (which are detergent-insoluble microdomains of intracellular vesicular membranes rich in cholesterol and sphingolipid) [11–13]. It has been reported that viral nonstructural proteins and both positive- and negative-sense HCV RNAs were localized distinctively in a fraction of lipid rafts when subgenomic HCV replicon cells were subjected to membrane flotation analysis [12]. On the other hand, recent studies have demonstrated that agents related to lipid metabolism affect the replication of genotype 1 HCV. Leu et al. [14] reported that polyunsaturated fatty acids exerted strong anti-HCV activity on a subgenomic HCV-1b replicon. Moreover, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins), which prevent cholesterol synthesis, have been shown to suppress replication of ge-

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nomic and subgenomic HCV-1b replicons [15, 16]. Even though the precise mechanism has not been defined, these agents may attenuate HCV replication through the destruction of lipid rafts, according to their pharmacological actions. If this is the mechanism, sphingomyelin, the remaining and essential component of lipid rafts, might play a role in HCV replication. With this in view, recent studies have demonstrated that a sphingomyelin synthesis inhibitor attenuated the replication of a subgenomic HCV-1b replicon in cultured cells [17] and the replication of genomic HCV-1 in a chimeric mouse model [18]. However, investigation of anti-HCV activity in these agents has been limited to genotype 1 HCV, and the combined effect of these agents has not been determined. If they do not target the HCV structure itself but exert their antiviral activity through destruction of the host's lipid raft, it would be plausible to speculate that they might be effective irrespective of the viral isolate, and the combined effect of these agents might be additive or synergistic.

In the present study, we investigated the role played by the sphingomyelin synthesis pathway and the mevalonate pathway in HCV replication, using a subgenomic HCV-1b replicon and the particle-producing cell culture HCV 2a model of JFH-1 HCV [19].

MATERIALS AND METHODS

Cell culture and HCV replicon. The human hepatoma cell lines Huh7 and Huh7.5.1 [20] were maintained in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal calf serum at 37°C in 5% CO_2. The subgenomic HCV replicon used was derived from Rep-Feo (genotype 1b) [21, 22], and a full-length genomic HCV RNA was derived from genotype 2a JFH-1 HCV [19]. Subgenomic or genomic HCV RNA was synthesized from replicon cDNA–harboring plasmids (pRep-Feo and pJFH-1) by means of T7 polymerase (RiboMax Large Scale RNA Production System; Promega) and transfected into these cells. For the subgenomic replicon, cell lines stably expressing the replicon were established (Huh7/Rep-Feo) in the presence of 500 μg/mL G418.

Reporter plasmids and luciferase assay. pISRE-TA-Rluc expressing the *Renilla* luciferase reporter gene under control of the IFN-stimulated response element (ISRE) was constructed by replacing the firefly luciferase gene with the *Renilla* luciferase gene of pISRE-TA-Luc, purchased from Invitrogen. Luciferase activity was quantified using the Bright-Glo or Dual-Luciferase assay system (both from Promega) and a luminometer (AB-2250; ATTO). Assays were performed in triplicate, and the results were expressed as mean ± SD percentages of the control values. QuantiLum recombinant luciferase (Promega) was used as the positive control for the analysis.

Reagents. The reagents used included myriocin (Biomol), IFN-α 2b (Santa Cruz Biotechnology), phytosphingosine hydrochloride (Sigma), 2-hydroxypropyl-β-cyclodextrin (2-HP-β-CyD; Sigma), and simvastatin (Cosmobio).

Northern blotting. Total cellular RNA was extracted from cells by means of Isogen (Wako). The RNA was separated by denaturing agarose-formaldehyde gel electrophoresis and transferred to a membrane from a NorthernMax kit (Ambion). The membrane was hybridized with a digoxigenin-labeled probe that was specific for the nonstructural replicon sequence. The signals were detected in a chemiluminescence reaction by using a digoxigenin detection kit (Roche) and were visualized by using an LAS-1000 imaging system (Fuji Film).

Western blotting. Ten micrograms of total cell lysate was separated using NuPAGE 4%–12% Bis-Tris gel (Invitrogen) and was blotted onto an Immobilon polyvinylidene difluoride membrane (Roche). The membrane was incubated with an anti-core monoclonal antibody (MAb; Affinity Bioreagents), an anti-NS3 MAb (Virogen), an anti-NS5A MAb (gift from Burckstummer, Robert Koch Institute), or a anti–β-catenin MAb (Sigma). Detection was done in a chemiluminescence reaction (ECL; Amersham).

Dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium (MTS) assays. To evaluate cytotoxicity, MTS assays were performed using a CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega), in accordance with the manufacturer's instructions.

Thin-layer chromatography (TLC). The lipid fraction of cells treated with myriocin was extracted using the method of Bligh and Dyer [23], and total lipids from the cells treated with myriocin were extracted with 3 mL of chloroform. The extracts were spotted onto silica gel TLC plates (Merck) and were chromatographed with chloroform-methanol-water (65:25:4 [vol/vol/vol]). The plate was visualized with a molybdenum spray.

Real-time reverse-transcription polymerase chain reaction (RT-PCR). TaqMan RT-PCR targeting the 5' untranslated region was used for the quantitation of intracellular genomic JFH-1 HCV RNA. The sequences of the sense and antisense primers and the TaqMan probe were 5'-TGCGGAACCGGTGAGTACA-3', 5'-CTTAAGGTTTAGGATTCGTGCTCAT-3', and 5'-(FAM)CACCCTATCAGGCAGTACCACAAGGCC(TAMRA)-3', respectively. The method has been described elsewhere [24].

Short interfering RNA (siRNA) analysis. The sequence encoding the LCB1 subunit of serine palmitoyltransferase (SPT) was selected as the target for siRNA (sense, 5'-AACAACAUCGUUUCAGGUCCUTT-3'; antisense, 5'-AGGGCCUGAAACGAUGUUGTT-3'). siRNA targeting enhanced green fluorescent protein (GFP) was used as the negative control (sense, 5'-CUUACGCUGAGUACUUCGATT-3'; antisense, 5'-UCGAAGUACUCAGCGUAATT-3'). (Underlined letters indicate deoxyribonucleotides.)

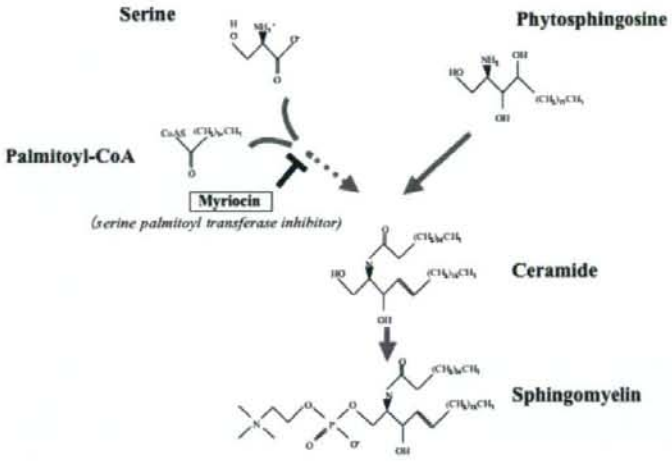

Figure 1. The sphingomyelin synthesis pathway. Serine palmitoyltransferase catalyzes the first committed step of sphingomyelin biosynthesis from serine and palmitoyl-coenzyme A (CoA). Myriocin inhibits the catalyzing activity of serine palmitoyltransferase. Phytosphingosine is known to work as a precursor of ceramide in both mammalian and fungal cells.

Statistical analyses. Statistical analyses were performed using Student's t test; statistically significant differences were defined as those for which $P < .05$.

RESULTS

Specific suppression of the replication of a subgenomic HCV-1b replicon by an inhibitor of sphingomyelin synthesis. To clarify the role played by the sphingomyelin synthesis pathway in HCV replication, we added myriocin, a specific inhibitor of SPT that catalyzes the first committed step of sphingomyelin biosynthesis (figure 1), to the medium of Huh7/Rep-Feo cells. The luciferase activity, reflecting replication of the subgenomic HCV-1b replicon, dropped to 37% and 21% of the control at myriocin concentrations of 100 and 1000 nmol/L, respectively (figure 2*A*, *upper panel*), but myriocin did not cause toxicity to the cultured cells (figure 2*A*, *lower panel*). The result indicates that the decrease in HCV replication is due to a specific suppressive effect of myriocin and not to the cytotoxicity of myriocin. Northern hybridization analysis also demonstrated a substantial reduction of the subgenomic HCV replicon RNA in Huh7/Rep-Feo cells treated with myriocin in a dose-dependent manner (figure 2*B*). Similarly, Western blot analysis demonstrated a decrease in HCV NS5A after treatment with myriocin (figure 2*C*).

No enhancement of ISRE promoter activity after myriocin treatment. To determine whether the effect of myriocin in suppressing the subgenomic HCV replicon was associated with the activation of IFN-stimulated genes, the ISRE–*Renilla* luciferase plasmid was transfected into Huh7/Rep-Feo cells, and these cells were cultured with various concentrations of myriocin. As a positive control for the enhancement of ISRE reporter activity, the ISRE–*Renilla* luciferase–transfected cells were cultured with IFN. Myriocin had no significant effect on ISRE promoter activity, whereas IFN significantly up-regulated ISRE activity (figure 2*D*, *upper panel*). In contrast, firefly luciferase activity in the Huh7/Rep-Feo cells, reflecting HCV replication, was inhibited by both IFN and myriocin in a dose-dependent manner (figure 2*D*, *lower panel*). These results demonstrate that the action of myriocin on HCV replication is independent of the IFN pathway.

Decrease in the sphingomyelin content of Huh7 cells after myriocin treatment. To clarify whether myriocin really inhibits the biosynthesis of sphingomyelin in Huh7 cells, we treated Huh7 cells with 100 nmol/L myriocin and analyzed the change in the cellular phospholipid composition by TLC. As demonstrated in figure 2*E*, the cellular sphingomyelin content decreased after myriocin treatment, but no significant change was observed in other cellular phospholipids.

Restoration of HCV replication by addition of phytosphingosine. To confirm that suppression of HCV RNA replication was due to depletion of sphingomyelin, we incubated replicon cells with phytosphingosine, a precursor of ceramide in mammalian and fungal cells, in the presence of myriocin. Treatment with phytosphingosine restored HCV replication in a dose-dependent manner (figure 2*F*, *upper panel*). On the other hand, phytosphingosine by itself did not have any effect on HCV replication (figure 2*F*, *lower panel*). This result indicates that inhibition of HCV replication was the direct result of depletion of sphingomyelin.

Suppression of HCV replication by knocking down SPT with siRNA. Next, we determined whether inhibition of SPT expression suppresses HCV replication by knocking down SPT with siRNA. As demonstrated in the upper panel of

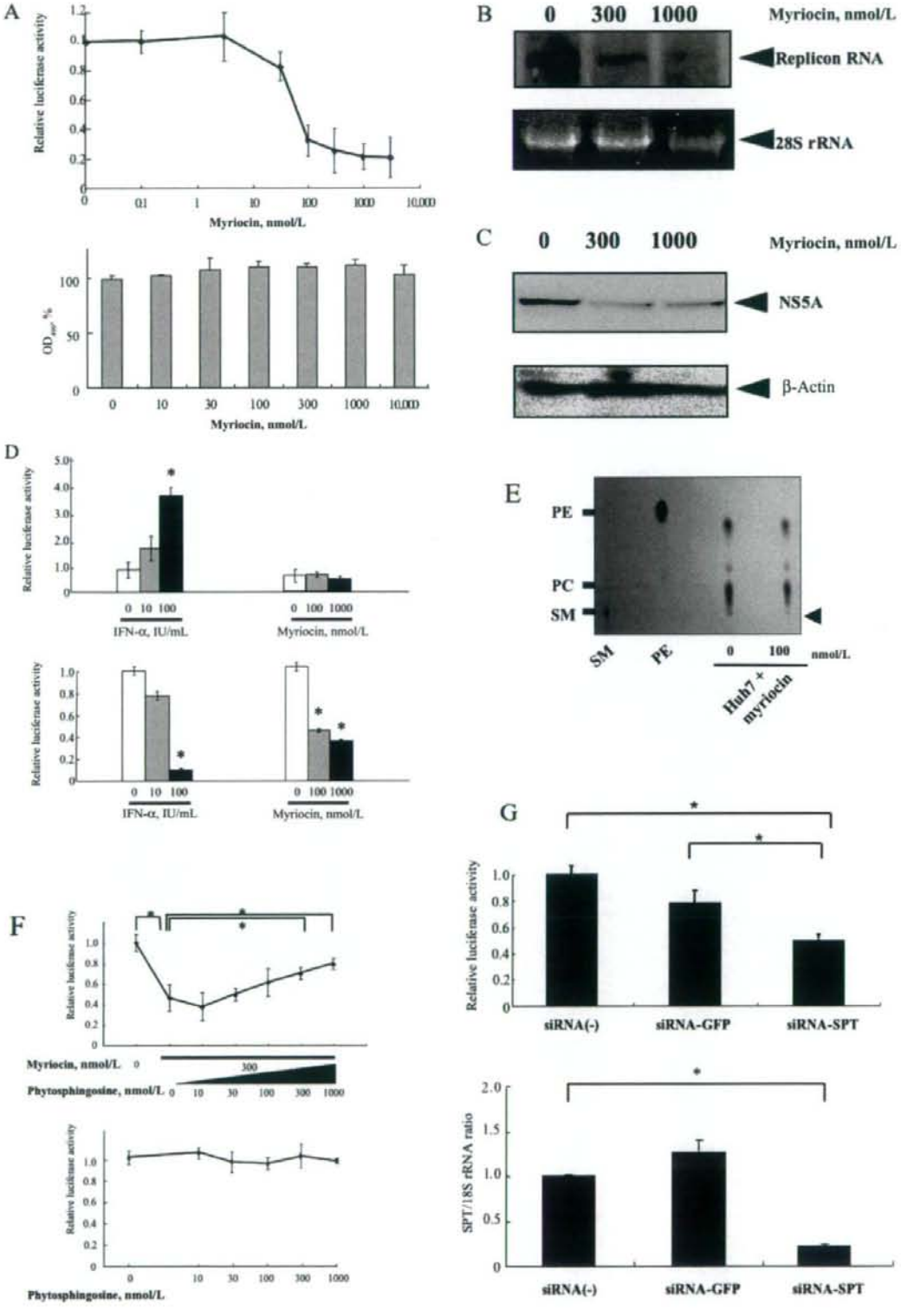
A
Relative luciferase activity
Myriocin, nmol/L
B
0 300 1000
Myriocin, nmol/L
Replicon RNA
28S rRNA
C
0 300 1000
Myriocin, nmol/L
NS5A
β-Actin
D
Relative luciferase activity
IFN-α, IU/mL
Myriocin, nmol/L
E
PE
PC
SM
SM PE 0 100 nmol/L
Huh7 + myriocin
F
Relative luciferase activity
Myriocin, nmol/L
Phytosphingosine, nmol/L
G
Relative luciferase activity
siRNA(-) siRNA-GFP siRNA-SPT
SPT/18S rRNA ratio

figure 2*G*, HCV replication was suppressed significantly by siRNA targeting SPT compared with no siRNA or siRNA targeting GFP (negative control). We confirmed with real-time PCR that the siRNA targeting SPT significantly decreased expression of SPT mRNA (figure 2*G*, *lower panel*). This result indicates that the SPT enzyme plays an important role in HCV replication.

Inhibition of the replication of a subgenomic HCV-1b replicon by an HMG-CoA reductase inhibitor (simvastatin). HMG-CoA reductase inhibitors have been reported to suppress replication of subgenomic and genomic HCV-1b replicons [15, 16]. Because cholesterol is another important component of lipid rafts, it may be speculated that depletion of cholesterol by HMG-CoA reductase inhibitors disrupts the lipid raft, affecting the ability of the HCV replicon to replicate in Huh7 cells. To confirm the effect of HMG-CoA reductase inhibitors on the subgenomic HCV-1b replicon, we examined the effect of simvastatin by means of Huh7/Rep-Feo cells. Cultures of Huh7/Rep-Feo cells with simvastatin at concentrations of 0–100 μmol/L showed a dose-dependent reduction of the subgenomic HCV-1b replicon (figure 3, *upper panel*). The MTS assay showed that treatment with simvastatin had no toxic effect on Huh7/Rep-Feo cells in the dose range used (figure 3, *lower panel*). These results demonstrated that simvastatin specifically suppressed replication of a subgenomic HCV-1b replicon. However, because recent studies showed that statins suppress HCV replication through inhibition of geranylgeranylation of certain proteins rather than inhibition of cholesterol synthesis [15], we also examined the effect on HCV replication of 2-HP-β-CyD, an agent known to deplete cholesterol directly from membranes. As demonstrated in figure 4*A*, 2-HP-β-CyD also suppressed HCV replication without cytotoxicity. To confirm that 2-HP-β-CyD did not inhibit firefly luciferase activity nonspecifically rather than by suppressing HCV RNA, we incubated recombinant firefly luciferase with various concentrations of 2-HP-β-CyD in the culture medium, and the medium was subjected to luciferase analysis. As demonstrated in figure 4*B*, 2-HP-β-CyD did not affect luciferase activity. These results indicate that cholesterol itself plays an important role in HCV replication.

Synergistic inhibitory effects of myriocin with IFN, simvastatin with IFN, and myriocin with simvastatin. We carried out the following assay to determine whether myriocin and IFN have a synergistic inhibitory effect on HCV replication. Huh7/Rep-Feo cells were treated with combinations of myriocin and IFN at various concentrations. The relative dose-inhibition curves of IFN were plotted for each fixed concentration of myriocin (0, 30, 100, and 300 nmol/L). As demonstrated in the upper panel of figure 5*A*, the curves shifted to the left with increasing concentrations of myriocin, demonstrating the synergy of the 2 drugs against the subgenomic HCV-1b replicon. Isobologram analysis also confirmed the synergy (figure 5*A*, *lower panel*). To determine whether this synergistic effect was associated with up-regulation of the IFN-stimulated gene responses, we investigated the combined effect of myriocin and IFN on ISRE activity. As demonstrated in figure 5*B* (*upper panel, right*), myriocin did not enhance the ISRE–*Renilla* luciferase activity induced by IFN, but

Figure 2. Specific inhibition of the replication of a subgenomic hepatitis C virus (HCV) genotype 1b replicon by myriocin. *A*, Inhibition of HCV replicon replication by myriocin. By use of Huh7/Rep-Feo cells expressing a selectable chimeric luciferase reporter Feo gene, the intracellular replication level of an HCV replicon was quantified on the basis of luciferase activity [22, 25]. Huh7/Rep-Feo cells were cultured with various concentrations of myriocin. After 96 h of treatment, the luciferase assay was performed, as described in Materials and Methods *(upper panel)*. In the dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium (MTS) assay, Huh7/Rep-Feo cells were cultured with various concentrations of myriocin for 96 h *(lower panel)*. Data are means ± SDs of triplicates from 2 independent experiments. *B*, Northern hybridization. Huh7/Rep-Feo cells were cultured with various concentrations of myriocin and harvested at 96 h after administration. Ten micrograms of total cellular RNA was electrophoresed in each lane. The membrane containing the HCV replicon RNA was hybridized using a digoxigenin-labeled probe specific for the replicon sequence *(upper panel)*, and 28S human ribosomal RNA (rRNA) was used as an internal control *(lower panel)*. Lane 1, no myriocin; lane 2, 300 nmol/L myriocin; lane 3, 1000 nmol/L myriocin. *C*, Western blotting. Ten micrograms of total cellular protein was electrophoresed in each lane. Anti-NS5A monoclonal antibody was used as the primary antibody to detect HCV proteins *(upper panel)*, and β-actin was used as an internal control *(lower panel)*. Lane 1, no myriocin; lane 2, 300 nmol/L myriocin; and lane 3, 1000 nmol/L myriocin. *D*, No enhancement of interferon (IFN)–stimulated response element (ISRE) promoter activity by myriocin. To investigate whether the effect of myriocin was associated with the activation of IFN-stimulated genes, the ISRE–*Renilla* luciferase plasmid was transfected into Huh7/Rep-Feo cells in the presence of myriocin. The upper panel demonstrates the ISRE–*Renilla* luciferase activity at 48 h after transfection. The lower panel demonstrates the firefly luciferase activity of the Huh7/Rep-Feo cells, reflecting HCV replication. Data are means ± SDs of triplicates from 2 independent experiments. $^*P < .05$. *E*, Decrease in the sphingomyelin (SM) content of Huh7 cells after myriocin treatment. The change in the cellular phospholipid content was analyzed by thin-layer chromatography. Huh7 cells were cultured alone or with 100 nmol/L myriocin for 96 h. PC, phosphatidylcholine; PE, phosphatidylethanolamine. *F*, Restoration of the HCV replication that was suppressed by myriocin after the addition of phytosphingosine. Huh7/Rep-Feo cells were cultured with myriocin alone or with various concentrations of phytosphingosine. The luciferase assay was performed after 72 h of treatment *(upper panel)*. Huh7/Rep-Feo cells were also cultured with phytosphingosine alone as indicated for 72 h *(lower panel)*. Data are means ± SDs of triplicates from 2 independent experiments. $^*P < .05$. *G*, Suppression of HCV replication by knocking down of serine palmitoyltransferase (SPT) with short interfering RNA (siRNA). Huh7/Rep-Feo cells were transfected with 10 nmol/L siRNA oligonucleotides targeting the LCB1 subunit of SPT or control siRNA targeting green fluorescent protein (GFP). The luciferase activity of the HCV replicon was measured 72 h after transfection *(upper panel)*. SPT mRNA expression at 72 h after siRNA transfection was analyzed by real-time polymerase chain reaction. The SPT mRNA level was measured relative to 18S rRNA *(lower panel)*. Values are shown as ratios to negative control levels and as the means ± SDs of triplicates from 2 independent experiments. siRNA(−), no siRNA. $^*P < .05$.

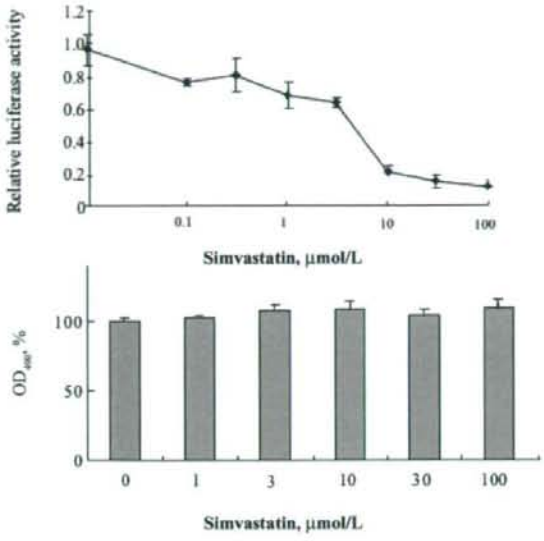

Figure 3. Inhibition of replication of a subgenomic hepatitis C virus genotype 1b replicon by simvastatin. Huh7/Rep-Feo cells were cultured with various concentrations of simvastatin, and the luciferase assay was performed after 48 h of treatment *(upper panel)*. The dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium assay was performed after Huh7/Rep-Feo cells were cultured with various concentrations of simvastatin for 48 h *(lower panel)*. Data are means ± SDs of triplicates from 2 independent experiments.

it significantly enhanced IFN-induced suppression of the firefly luciferase activity reflecting HCV replication *(lower panel, right)*. This demonstrated that the synergistic effect was not caused by up-regulation of the IFN-stimulated genes. We also assessed the synergy of simvastatin with IFN and of myriocin with simvastatin. In each case, the 2 drugs showed synergistic effects at the concentrations indicated (figure 5*C* and 5*D*). In all cases, the MTS reduction values at the drug concentrations used in this assay did not show any significant decrease (data not shown). These results indicate that the synergistic effects on HCV replication of IFN with myriocin, IFN with simvastatin, and myriocin with simvastatin were exerted through their pharmacological effects and were not due to the augmentation of cytotoxicity.

Suppression of JFH-1 HCV replication by myriocin and simavastatin. The experiments described thus far were done using the subgenomic HCV-1b replicon system. Recently, Wakita et al. [19] established an infectious HCV model in cultured cells. This system, known as the JFH-1 system and based on genotype 2a HCV, secretes viral particles into the medium, and the medium is infectious for chimpanzees. This JFH-1 system completely mimics HCV infection in vivo and is considered more suitable for analyzing the effect of drugs. Therefore, we examined the effect of myriocin and simvastatin using the JFH-1 system. Huh7.5.1/JFH-1 HCV cells were cultured for 96 h with 1000 nmol/L myriocin, 10 μmol/L simvastatin, 1000 IU/mL IFN, and a combination of 1000 nmol/L myriocin and 10 μmol/L simvastatin. The intracellular JFH-1 HCV RNA titer was analyzed using real-time RT-PCR. As demonstrated in figure 6*A*, intracellular JFH-1 HCV RNA treated with myriocin or simvastatin decreased to 60% of control in 96 h, demonstrating that the inhibitory effect of myriocin and simvastatin on replication was not restricted to the subgenomic HCV-1b replicon. When both agents were used in combination, JFH-1 HCV RNA also

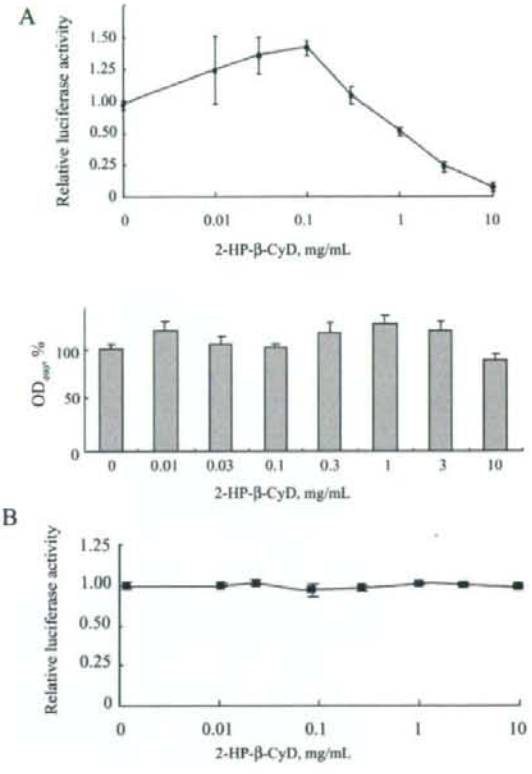

Figure 4. Inhibition of replication of a subgenomic hepatitis C virus genotype 1b replicon by 2-hydroxypropyl-β-cyclodextrin (2-HP-β-CyD). *A*, Huh7/Rep-Feo cells cultured with various concentrations of 2-HP-β-CyD for 48 h. The luciferase assay was performed after 48 h of treatment *(upper panel)*. The dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium assay was performed after Huh7/Rep-Feo cells were cultured with various concentrations of 2-HP-β-CyD for 48 h *(lower panel)*. Data are means ± SDs of triplicates from 2 independent experiments. *B*, Recombinant firefly luciferase incubated with various concentrations of 2-HP-β-CyD in the culture medium at 37°C for 48 h. The medium was collected and subjected to luciferase analysis. Data are means ± SDs of triplicates from 2 independent experiments.

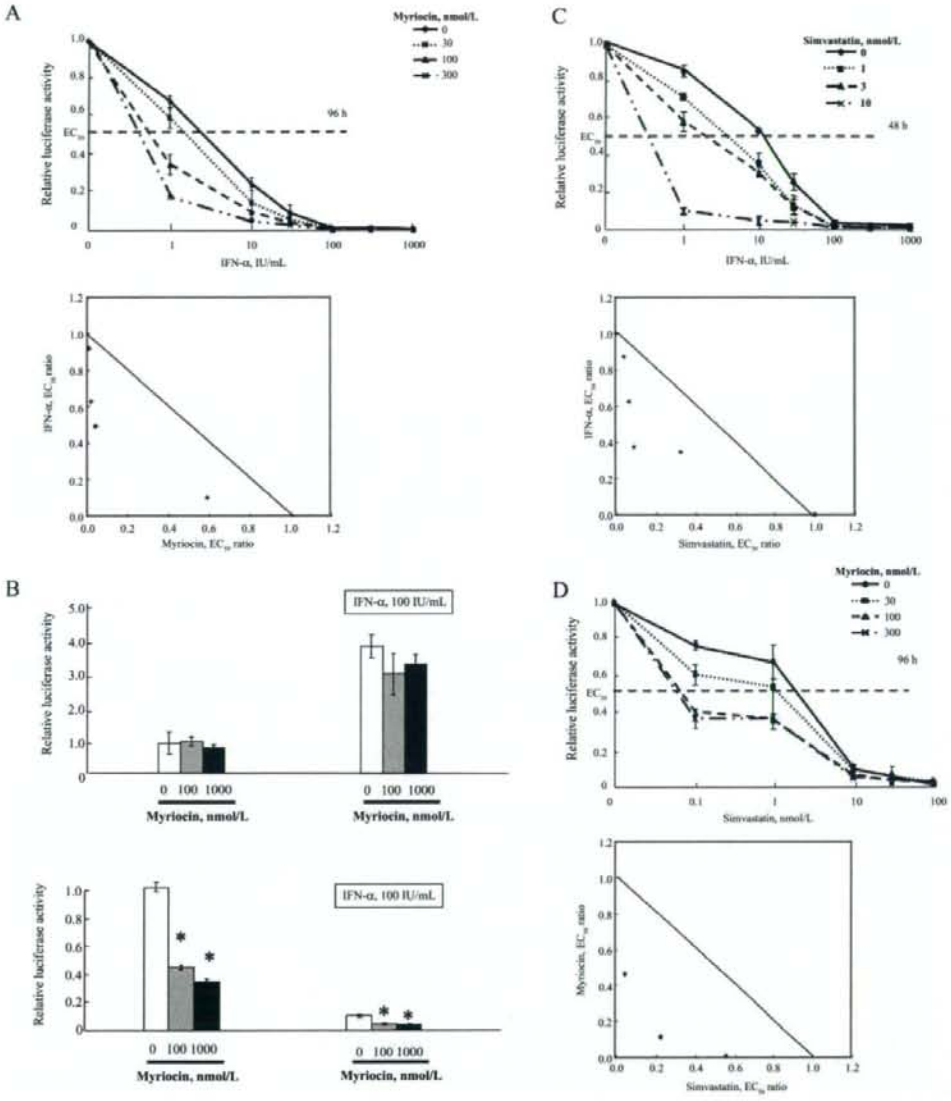

Figure 5. Synergistic inhibitory effects of myriocin with interferon (IFN), simvastatin with IFN, and myriocin with simvastatin. *A*, Synergistic inhibitory effect of myriocin with IFN on hepatitis C virus replication. Huh7/Rep-Feo cells were treated with combinations of myriocin and IFN at various concentrations. The upper panel shows the relative dose-inhibition curves of IFN plotted for each fixed concentration of myriocin (0, 30, 100, and 300 nmol/L). The lower panel shows the isobologram analysis for the combination of myriocin with IFN. *B*, IFN-stimulated response element (ISRE) promoter activity induced by a combination of myriocin with IFN. Huh7/Rep-Feo cells transfected with ISRE–*Renilla* luciferase were cultured with various concentrations of myriocin alone *(left)* or with 100 IU/mL IFN *(right)*. The upper panel demonstrates the ISRE–*Renilla* luciferase activity at 48 h after transfection. The lower panel demonstrates the firefly luciferase activity of the Huh7/Rep-Feo cells, reflecting hepatitis C virus (HCV). Data are means ± SDs of triplicates from 2 independent experiments. $^*P < .05$. *C*, Synergistic inhibitory effect of simvastatin with IFN on HCV replication. *D*, Synergistic inhibitory effect of simvastatin and myriocin on HCV replication.

decreased to almost 60% of the control at 48 and 96 h after treatment. However, no evident synergistic inhibitory effect was observed (figure 6*A*). To clarify the inhibitory effect of myriocin on JFH-1 HCV, we performed Western blot analysis for JFH-1 HCV proteins. As demonstrated in figure 6*B*, a substantial decrease in the core and NS3 proteins of JFH-1 HCV was observed 96 h after treatment with myriocin, confirming the RT-PCR results (figure 6*B*).

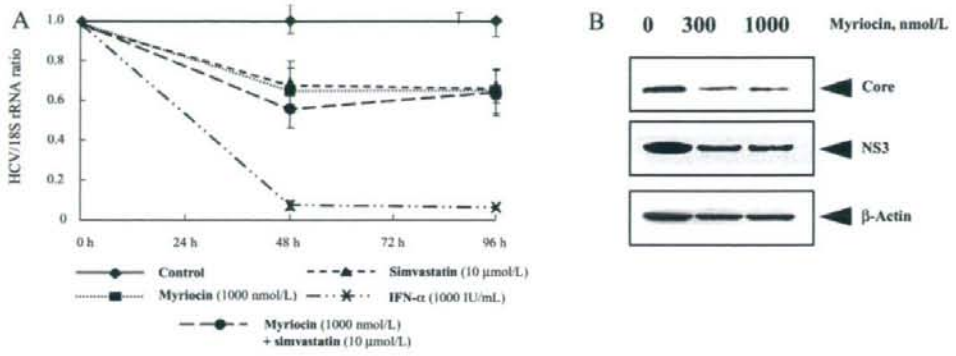

Figure 6. Suppression of JFH-1 hepatitis C virus (HCV) replication by myriocin and simvastatin. *A*, Cells containing JFH-1 HCV treated for 96 h with 1000 nmol/L myriocin, 10 μmol/L simvastatin, 1000 IU/mL IFN, or a combination of 1000 nmol/L myriocin and 10 μmol/L simvastatin. The cells were collected at 48 and 96 h, and the JFH-1 HCV RNA level relative to 18S rRNA was analyzed by real-time polymerase chain reaction. Values are shown as the ratios to negative control values (cells receiving no treatment) and as means ± SDs. *B*, Western blotting. Cells containing JFH-1 HCV were treated with 300 or 1000 nmol/L of myriocin and harvested at 96 h after administration. Ten micrograms of total cellular protein was electrophoresed in each lane. Anti-core monoclonal antibody (MAb) and anti-NS3 MAb were used as the primary antibodies to detect JFH-1 HCV proteins. β-Actin was detected as an internal control. Lane 1, no myriocin; lane 2, 300 nmol/L myriocin; and lane 3, 1000 nmol/L myriocin.

DISCUSSION

In the present study, we demonstrated that the sphingomyelin synthesis inhibitor myriocin suppressed not only replication of a subgenomic HCV-1b replicon but also replication of the JFH-1 strain of infectious genotype 2a HCV. We also demonstrated that simvastatin suppressed replication of both a subgenomic HCV-1b replicon and JFH-1 HCV. When a subgenomic HCV-1b replicon was used, the anti-HCV activity of both myriocin and simvastatin was enhanced synergistically with IFN. Moreover, when myriocin and simvastatin were used together, their anti-HCV activity was enhanced synergistically.

What is the mechanism by which myriocin suppresses viral replication? Because myriocin is a specific inhibitor of SPT, which catalyzes the first committed step of sphingomyelin biosynthesis, we speculated that myriocin exerts its action by inhibiting production of downstream substrates, especially sphingomyelin. The findings that siRNA targeted against SPT decreased HCV replication and that HCV replication was restored by addition of phytosphingosine, a precursor of sphingomyelin, demonstrated that the effect was specific to SPT activity. Moreover, the fact that treatment of Huh7 cells with myriocin did not enhance the ISRE promoter activity indicated that the inhibitory effects of myriocin were independent of those of IFN. It is known that intracellular replication of most RNA viruses occurs on certain membrane structures—including the endoplasmic reticulum, the Golgi apparatus, endosomes, and lysosomes—by making replication complexes at these sites [5–7]. For HCV, it has been reported by several groups that in vitro replication activity is located in the membrane fractions of cultured cells [26–28]. In addition, newly synthesized HCV RNA and the nonstructural proteins in replicon cells were colocalized in detergent-resistant membrane structures, most likely lipid rafts [18]. Caveolin-2, a lipid raft protein, was also shown to colocalize with the nonstructural proteins [18]. According to these findings, the HCV replication complex machinery is considered to form on a lipid raft. Therefore, because sphingomyelin is the major component of the lipid raft, it is plausible to speculate that myriocin disrupted lipid raft formation and inhibited HCV replication.

Cholesterol is another major component of lipid rafts and might also be targeted for anti-HCV therapy. Because cholesterol is synthesized in the mevalonate pathway, an inhibitor of the pathway might act to disrupt lipid rafts. In accordance with this concept, statins, which are HMG-CoA reductase inhibitors, already have been reported to suppress the replication of genomic and subgenomic HCV-1b replicons [15, 16]. In the present study, we also confirmed that simvastatin suppressed replication of a subgenomic HCV-1b replicon without toxicity. Moreover, we showed for the first time that the suppressive effect was also observed in an infectious HCV-2a model of JFH-1 HCV. Meanwhile, recent studies found that the effect of statins was attributable to inhibition of geranylgeranylation rather than depletion of cholesterol, because addition of geranylgeraniol rescued HCV suppression induced by statins [15]. However, although geranylgeranylation might play a role in HCV regulation, the importance of cholesterol itself has not yet been determined. To clarify further the role played by cholesterol in HCV replication, we investigated the effect of 2-HP-β-CyD, which is known to deplete cholesterol directly from cells. As demonstrated in figure 4, specific suppression of HCV replication by 2-HP-β-CyD indicated the importance of cholesterol itself for HCV replication. It is unlikely that these agents suppressed replication of the subgenomic replicon through inhibi-

tion of encephalomyocarditis virus internal ribosome entry site (EMCV-IRES) activity, because they also significantly suppressed replication of a full-length genomic HCV (JFH-1 HCV) that does not include EMCV-IRES (figure 6*A*; data for 2-HP-β-CyD not shown).

Although we observed an inhibitory effect of myriocin and simvastatin on both the subgenomic HCV-1b replicon and JFH-1 HCV, there was a difference in efficacy between the 2 HCV systems; the subgenomic HCV-1b replicon was more sensitive to and was more strongly inhibited by either agent alone or in combination, compared with JFH-1 HCV. This result was unexpected, because we had speculated that these agents might be effective irrespective of the viral isolate if these agents targeted not the virus itself but rather host factors, such as lipid rafts. However, there are several differences between these 2 systems, and we cannot directly compare the results. In particular, the subgenomic HCV replicon lacks viral structural proteins and has only an HCV RNA intracellular replication step, whereas JFH-1 HCV includes all steps of the HCV life cycle. We do not know the precise target of the agents, and further studies are still needed.

Is it really possible to use these agents in clinical HCV treatment? Especially because statins have been used in the treatment of hyperlipidemia for many years worldwide with proven safety, it would be ideal if we could use statins as one therapeutic application for anti-HCV therapy. Most recently, O'Leary et al. [29] undertook a human pilot study and treated 10 patients with atorvastatin for 12 weeks; they reported that there was no statistically significant change in HCV RNA levels compared with pretreatment levels. The reason for the discrepancy between in vitro and in vivo findings is unknown. However, as also discussed by O'Leary et al., the most plausible explanation for this discrepancy is that the plasma concentrations of atorvastatin after a conventionally approved dose were unlikely to reach those found to be effective in cell culture medium. According to their calculations, to inhibit HCV RNA replication the plasma atorvastatin concentration should be 3 logs higher than that achieved by a conventional dose. However, even though it would be difficult to inhibit HCV RNA replication with statins alone, a clinical antiviral effect might be still achieved if statins were used in combination with IFN (or myriocin), because a synergistic effect was observed in our in vitro study. To determine the synergistic effect in vivo, however, further clinical trials are needed. On the other hand, although promising in vitro, myriocin has not yet been used for human clinical diseases, and its safety has not been established. However, in chimeric mice, the plasma myriocin concentration equivalent to culture medium effectively inhibited HCV RNA replication, and drug toxicity was not observed at this concentration [30]. This finding suggested the possibility that myriocin could be used in vivo, although further studies are needed.

In conclusion, we have demonstrated that inhibition of the sphingomyelin synthesis pathway and the mevalonate pathway both effectively suppressed HCV replication in vitro, indicating that lipid metabolism could be an important target for new anti-HCV therapies.

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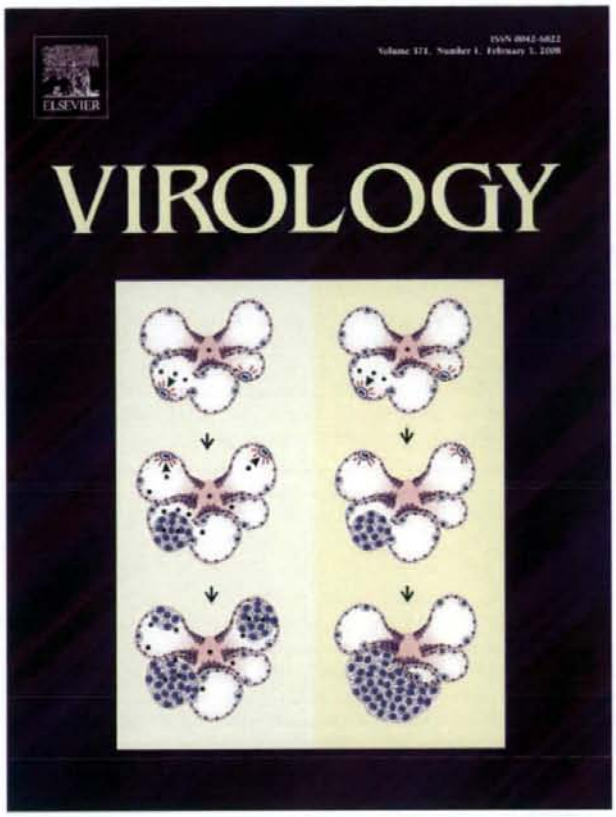

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Development of plaque assays for hepatitis C virus-JFH1 strain and isolation of mutants with enhanced cytopathogenicity and replication capacity

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Abstract

HCV culture *in vitro* results in massive cell death, which suggests the presence of HCV-induced cytopathic effects. Therefore, we investigated its mechanisms and viral nucleotide sequences involved in this effect using HCV-JFH1 cell culture and a newly developed HCV plaque assay technique. The plaque assay developed cytopathic plaques, depending on the titer of the inoculum. In the virus-infected cells, the ER stress markers, GRP78 and phosphorylated eIF2-alpha, were overexpressed. Cells in the plaques were strongly positive for an apoptosis marker, annexin V. Isolated virus subclones from individual plaque showed greater replication efficiency and cytopathogenicity than the parental virus. The plaque-purified virus had 9 amino acid substitutions, of which 5 were clustered in the C terminal of the NS5B region. Taken together, the cytopathic effect of HCV infection involves ER-stress-induced apoptotic cell death. Certain HCV genomic structures may determine the viral replication capacity and cytopathogenicity.

Keywords: HCV-JFH1; HCV cell culture; Plaque assay; ER stress; Unfolded protein responses; Apoptosis; NS5B RNA-dependent RNA polymerase

Introduction

Molecular analyses of the HCV life cycle, virus–host interactions, and mechanisms of liver cell damage by the virus are not understood completely, mainly because of the lack of cell culture systems. These problems have been partly overcome by the development of the HCV subgenomic replicon (Lohmann et al., 1999) and HCV cell culture systems (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). The HCV-JFH1 strain, which is a genotype 2a clone derived from a Japanese fulminant hepatitis patient that can replicate efficiently in Huh7 cells (Kato et al., 2003; Kato et al., 2001), has contributed to the establishment of the HCV cell culture system. Furthermore, the Huh7-derived cell lines, Huh-7.5 cells, Huh-7.5.1, and Lunet cells allow production of higher viral titers and have a higher permissiveness for HCV (Koutsoudakis et al., 2007; Lindenbach et al., 2005; Zhong et al., 2005). The HCV-JFH1 cell culture system now allows us to study the complete HCV life cycle: virus–cell entry, translation, protein processing, RNA replication, virion assembly, and virus release.

HCV belongs to the family *Flaviviridae*. One of the characteristics of the *Flaviviridae* is that they cause cytopathic effects (CPE). The viruses have positive strand RNA genomes of ~10 kilobases that encode a polyprotein of ~3000 amino acids.

Abbreviations: HCV, hepatitis C virus; IFN, interferon; CPE, cytopathic effect; ER, endoplasmic reticulum; UPR, unfolded protein response; PFU, plaque-forming unit; FFU, focus-forming unit; RdRp, RNA-dependent RNA polymerase.

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The protein is post-translationally processed by cellular and viral proteases into at least 10 mature proteins. The viral nonstructural proteins accumulate in the ER and direct genomic replication and viral protein synthesis (Bartenschlager and Lohmann, 2000; Jordan et al., 2002; Mottola et al., 2002). It has been reported that Japanese encephalitis virus (JEV), bovine viral diarrhea virus (BVDV), and dengue viruses (DEN) cause apoptotic cell death (Despres et al., 1996; He, 2006; Jordan et al., 2002; Su et al., 2002). In addition, certain amino acid substitutions in the viral structural or nonstructural proteins affect the replication and cytopathogenicity of these viruses substantially (Blight et al., 2000; Maekawa et al., 2004; Mendez et al., 1998). It has been recently reported that HCV-JFH1-transfected Huh-7.5.1 cells died when all of the cells were infected and intracellular HCV-RNA reached maximum levels (Zhong et al., 2006). These findings suggest HCV-induced cytopathogenicity. However, the mechanisms have not been well documented.

In the present study, we investigated the cellular effects of HCV infection and replication using the HCV-JFH1 cell culture system. Here, we report that HCV-JFH1-transfected and infected cells show substantial CPE that are characterized by massive apoptotic cell death with the expression of several ER stress-induced proteins. Taking advantage of the CPE, we developed a plaque assay for HCV in cell culture and isolated subclones of HCV that showed enhanced replication and cytopathogenicity. We have demonstrated that these viral characters were determined by mutations at certain positions in the structural and nonstructural regions of the HCV genome, especially the NS5B region.

Results

Production of infectious HCV-JFH1 by JFH1-RNA transfected cells

After transfection of HCV-JFH1 RNA into Huh-7.5.1 cells, intracellular HCV RNA and HCV antigen were continuously detectable in the cell culture (Fig. 1A). Furthermore, the culture supernatant from the transfected cells was positive for core protein, which reached maximum levels at 14 days post-transfection and was continuously detectable during the cell culture (Fig. 1A, black bar). The culture supernatant was readily infectable to naive Huh-7.5.1 cells (data not shown). Immunofluorescence assay showed that 48% of the JFH1-RNA-transfected cells and 42% of the virus-infected cells were positive for HCV core protein. These results demonstrate that the transcript of HCV-JFH1 clone replicates efficiently and produces infectious virus particles in cells, as reported previously (Wakita et al., 2005; Zhong et al., 2005).

Hepatitis C virus infection induced cytopathic effects in vitro

By the seventh day post-transfection, the production of virus decreased concomitant with massive cell death and then cell growth gradually recovered. At 14–16 days post-transfection, the levels of HCV-RNA and core antigen reached maximum (Fig. 1). In the JFH1 mutants JFH1/GND and JFH1/ΔE1-E2-RNA-transfected Huh-7.5.1 cells, the viral replication and host cell death were not observed. The massive cell death after HCV-

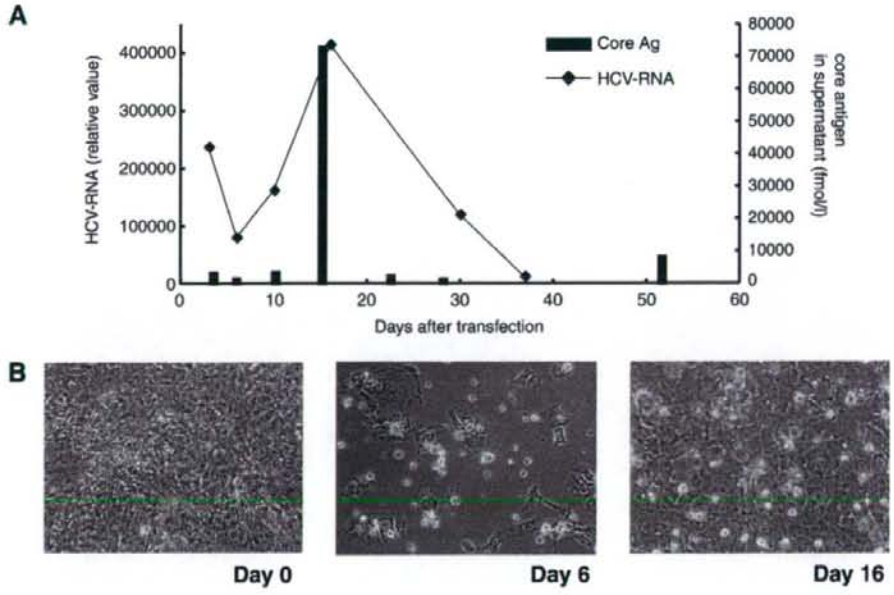

Fig. 1. Replication of HCV-JFH1 RNA in JFH1-transfected and infected Huh-7.5.1 cells. (A) Levels of HCV-RNA in JFH1 RNA-transfected cells. After transfection of the *in vitro* transcribed JFH1-RNA into Huh-7.5.1 cells, total cellular RNA was isolated on indicated days and quantified by real-time RT-PCR. Furthermore, the culture supernatant of JFH1-RNA transfected Huh-7.5.1 cells was collected on the days indicated and the levels of core antigen in the culture supernatant were measured (black bar). (B) HCV-JFH1-transfected Huh-7.5.1 cells (the left panel, day 0; the middle panel, day 6; the right panel, day 16).

JFH1 transfection led us to suspect the occurrence of CPE, produced in host cells by HCV infection and replication. A plaque assay was performed (see Materials and methods) to investigate the morphological CPE following HCV-JFH1 infection. Culture supernatants from JFH1-transfected cells were diluted serially and inoculated onto uninfected Huh-7.5.1

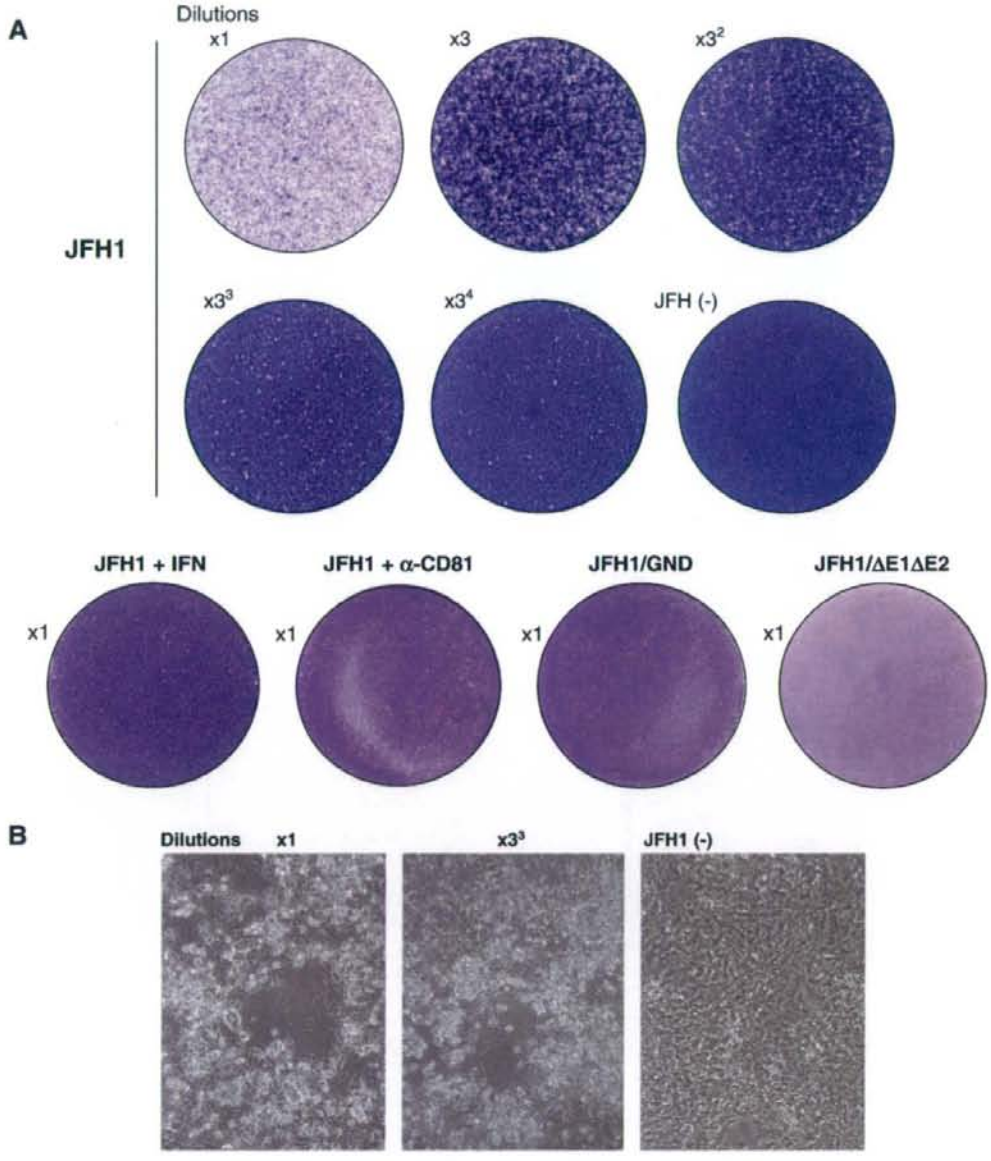

Fig. 2. The cytopathic effects of HCV-JFH1 *in vitro*. (A) Plaque assay. Upper panel, Huh-7.5.1 cells were seeded in collagen-coated 60-mm-diameter plates at density of 4×10^5 cells per plates and were incubated at 37 °C under 5.0% CO_2 (as described above). After overnight incubation, HCV-infected culture supernatants were serially diluted in a final volume of 2 ml per plates and transferred onto the cell monolayers. After ~5 h of incubation, the inocula were removed and the infected cells were overlaid with 8 ml of culture medium containing 0.8% methylcellulose and incubated under normal conditions. After 7 days culture, formation of cytopathic plaque was visualized by staining with 0.08% crystal violet solution. Lower panel, JFH1 + IFN; after infection of the virus supernatant, the cells were cultured in the presence of 50 U/ml interferon-alpha. JFH1 + a-CD81, Huh-7.5.1 cells were pretreated with 10 μg/plate of anti-CD81 antibody. After incubation at 37 °C for 30 min, anti-CD81 was removed, the cells were washed with PBS, and the HCV-JFH1 culture supernatant was transferred. After ~5 h incubation, the supernatant was removed and the infected cells were overlaid with 8 ml of culture medium containing 0.8% methylcellulose and controls for the plaque assay were also performed with the JFH1/GND or JFH1/ΔE1-E2 culture supernatant. (B) The cytopathic plaques were observed by phase-contrast microscopy at day 7 after HCV-JFH1 infection.

cells. The cells were subsequently cultured in medium containing agarose. Almost 10 days after the inoculation, viable cells were stained and plaques were visualized (Fig. 2A, upper panel). HCV-inoculated cell cultures developed plaques as unstained areas that were accompanied by round cells in the periphery (Fig. 2B). The formation of cytopathic plaques was not observed in a parental Huh7 cell line (data not shown). Immunocytochemistry of the foci revealed the presence of HCV core-positive cells surrounding the cytopathic plaques (Fig. 3A). Culture of the HCV-inoculated cells in the presence of interferon-alpha (50 U/ml) completely abolished the formation of plaques (Fig. 2A, lower panel). Uninfected Huh-7.5.1 cells (Fig. 2A, upper panel), Huh-7.5.1 cells treated with anti-CD81 antibody before HCV-JFH1 infection and JFH1/GND or JFH1/ΔE1-E2-transfected cell cultures did not develop plaques (Fig. 2A, lower panel). These findings suggest that HCV-infected cells develop cytopathic plaques depending on the quantity of the inoculums and that HCV replication, viral protein expression and the propagation of viral particles were the features of these plaques.

HCV-JFH1 infection induced host-cell apoptosis

We next determined whether the cytopathic effects of HCV-JFH1 replication include process of apoptotic cell death. Cells including plaques were double-stained with annexin V-FITC and PI. The ligand of annexin V, phosphatidylserine, is normally confined to the cytoplasmic leaflets of the plasma membrane. In the early phase of apoptosis, phosphatidylserine is exposed on the outer surface of the plasma membrane, which enables detection of FITC-labeled annexin V. As shown in Fig. 4, the fluorescence of annexin V was observed in the cells around the plaques. Foci of apoptotic cells were scattered in the plaques. On the other hand, the expression of annexin V was slightly detectable in the subgenomic replicon-harboring cells, though they were at the same level as the uninfected Huh-7.5.1 cells and the cell death was not observed. Therefore, the cells that express HCV subgenomic replicons did not induce apoptotic cell death. These findings demonstrate that the cytopathic effects of HCV replication and the particle formation induce apoptotic cell death.

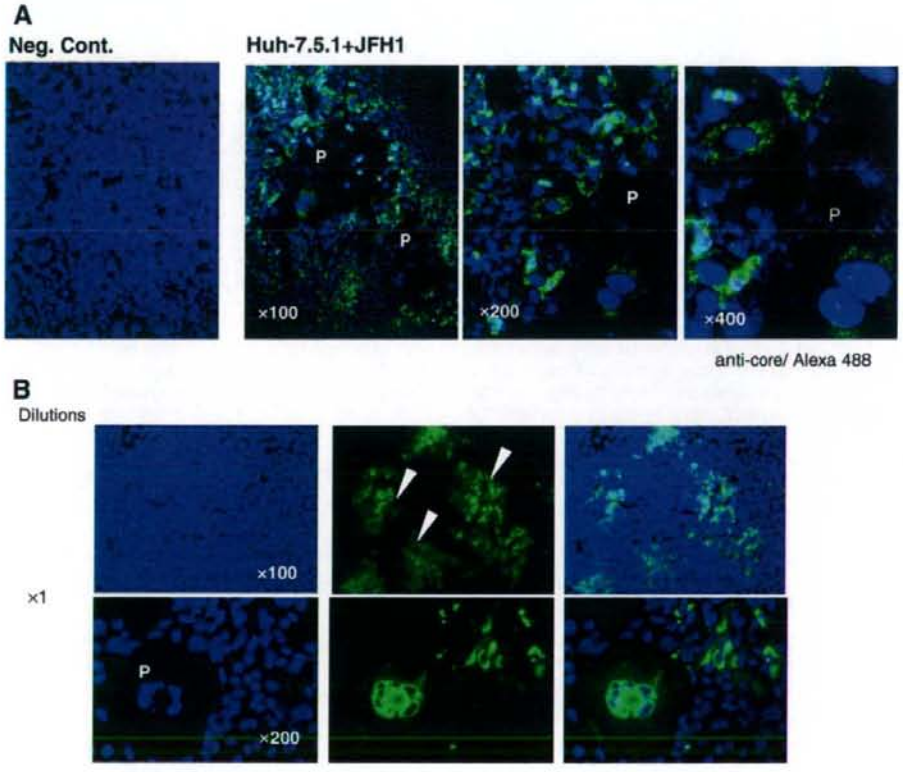

Fig. 3. Immunofluorescence detection of HCV core protein in cytopathic plaques. (A) The HCV-JFH1 culture supernatant was transferred onto uninfected Huh-7.5.1 cells, plated on 22 mm-round micro cover glasses in 60-mm-diameter plates at density of 2×10^5 cells per plate. After ~5 h incubation, the supernatant was replaced with medium containing 0.8% methylcellulose. Immunocytochemistry was performed 12 days after infection. A 'P' indicates a cytopathic plaque. (B) Immunofluorescence detection of HCV-positive foci and cytopathic plaques. The HCV-JFH1 culture supernatant was transferred at various dilutions onto uninfected Huh-7.5.1 cells. After ~5 h incubation, the supernatant was removed and the infected cells were cultured in 60-mm-diameter plate with medium containing 0.8% methylcellulose. Immunocytochemistry was performed 5 days after infection using mouse anti-core antibody. The infectivity and cytotoxicity were quantified by counting HCV-positive foci (FFU/ml) and cytopathic plaque (PFU/ml) respectively. White arrowheads indicate HCV-positive foci.